

Development of a quantitative technique to assay changes in markers of neural  
plasticity in response to intermittent theta burst stimulation

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“I declare that this report is my own original work and that contributions of others  
have been duly acknowledged.”

Signature \_\_\_\_\_

Date \_\_\_\_\_

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## **Abstract**

Delivery of intermittent theta burst stimulation (iTBS) can modulate cortical excitability, and if delivered prior to motor training, it can facilitate performance. These effects suggest that iTBS can induce long term potentiation (LTP) like changes within the associated brain areas. However, currently there is a lack of physiological evidence for such processes. Evidence for molecular changes obtained using animal models of iTBS is inconclusive, and methodologically varied. In addition, the use of human sized coil in laboratory rodents further compromises translational merits of obtained findings. Present study is conducted as part of a larger project that uses translational approach to study neurophysiological mechanisms of iTBS in rodents with species specific stimulation coil. Using immunohistochemical analysis of mouse brain sections, changes in presynaptic LTP marker Synaptophysin were investigated in eight animals that demonstrated increased forelimb reaching accuracy over 10 days in a skilled-motor-task after receiving iTBS. Changes in expression of Synaptophysin were compared between three groups (iTBS, sham, handling control), within three brain regions (primary motor cortex, dorsal striatum, piriform cortex). In all three regions, there were no significant differences found between the three groups, suggesting that after 10 days of training, homeostatic process of synaptic scaling may have taken place.

## **Neuroplasticity and its Benefits in Therapeutic Settings**

It is now widely understood that brain circuitry does not remain static throughout life; rather, it changes in response to environmental demands. This ability of the nervous system to adapt to intrinsic and extrinsic demands is referred to as neuroplasticity (Pascual-Leone, Amedi, Fregni, & Merabet, 2005). It is an ongoing process occurring throughout the lifespan that can be observed at many levels, from molecular and cellular, to systemic and behavioural. Early in life, neuroplasticity is essential for establishment of functional brain circuitry, and it is argued that later in life brain plasticity is crucial to normal functioning, as it forms basis of learning and memory, and underpins crucial rehabilitative responses to brain trauma (Pascual-Leone et al., 2005). There is an ongoing interest in understanding the mechanisms of neural plasticity in an effort to help restore normal functioning in individuals affected by neurological, neurodegenerative, psychiatric or mental disorders. A recent review of neuroplastic adaptations in clinical practice identified two existing ways of harnessing benefits of brain plasticity: a) delivery of therapies promoting use-dependant neuroplasticity; and b) brain stimulation techniques that induce neuroplasticity passively (Cramer et al., 2011).

One such passive brain stimulation technique, repetitive transcranial magnetic stimulation (rTMS), has been found to modify cortical plasticity by means of the repeated application of extrinsic transient magnetic fields. The improvements in symptoms following rTMS have been investigated in a wide range of clinical conditions, and currently, rTMS is approved as a therapeutic option for treatment of major depressive disorder, migraine and nerve pain (Cramer et al., 2011). However, there are inconsistencies reported in the effects of stimulation between and within individuals (Stockel, Summers, & Hinder, 2015), compromising the effectiveness of

the treatment (Ridding & Ziemann, 2010). In addition, the neurophysiological mechanisms of observed rTMS-induced behavioural modifications are still largely unknown, since most of the research focus is on investigating the functional consequences of rTMS-induced change, rather than its underlying neural mechanisms (Pascual-Leone et al., 2011).

It could be argued that in order to fully harness any therapeutic benefits of rTMS, the underlying cellular and molecular mechanisms facilitating the neuroplastic response must be fully understood. Due to the invasive nature of techniques employed to study cellular and molecular physiology, studies investigating these processes traditionally involve animal models. Use of animal models for study of human conditions is possible due to many shared homological features of the central nervous system (e.g. similarities in cellular structure, connectivity, organisation) (Kirkcaldie, 2012). Experimental animal models are therefore often used to guide the development of novel treatments and intervention for both physical and mental health conditions found in humans (Gallagher & Rapp, 1997; Gotz & Ittner, 2008; L. W.-H. Lee, 2008; Y. Xu, Barish, Pan, Ogle, & O'Donnell, 2012).

The aim of the present study is to extend the scientific enquiry into the therapeutic potential of rTMS, by investigating its neurophysiological effects at cellular and molecular level using genetically identical laboratory mice. In particular, the similarities and/or differences in cellular responses to rTMS induced plasticity, are compared to known molecular changes associated with use dependant plasticity observed following motor skill acquisition.

## **Motor Learning Paradigms Used to Study Neuroplasticity**

Motor learning tasks are widely used to study processes of neuroplasticity within the central nervous system. The acquisition of a new motor skill is demonstrated by enhancement of performance on a motor task over time, which, depending on the task, could be measured as changes in accuracy, speed, reaction time, or movement variability (Dayan & Cohen, 2011). Sequential learning tasks, during which a novel motor sequence is acquired through repeated training, are successfully used to study learning induced plasticity in both humans and animals. Human studies often involve learning of a novel fine motor finger sequence, and offer insights into behavioural and systemic changes that occur during learning of this task (Pascual-Leone et al., 2005). Animal models allow for application of more invasive investigations of cellular and molecular mechanisms that underlie these functional changes. The motor learning paradigm of skilled reaching is commonly used with rodents, as it was found to have a high translational merit with fine motor learning tasks in humans (Whishaw, Pellis, & Gorny, 1992). During skilled reaching training, animals are trained over several days to retrieve a food pellet in a way that forces them to use muscles of their preferred rostral forelimb in a novel way, by precisely controlling movement of their digits and wrist (Whishaw & Pellis, 1990).

### **Fast Motor Learning**

The Process of motor skill acquisition typically occurs in two stages, an early stage categorised as fast motor learning, and later stage of slow motor learning (Dayan & Cohen, 2011). Fast learning is characterised by association formation, and an initial rapid increase in task performance (Karni et al., 1998). The length of this stage is task dependant, e.g. when learning to play violin, fast learning can take several months, however fast learning of a simple four component key press routine

may occur over a few days. Using functional neuroimaging methods, it was demonstrated that fast learning of a sequential motor task is associated with changes in activity in several cortical and subcortical regions, such as dorsolateral prefrontal cortex, primary motor cortex, pre-supplementary motor area, supplementary motor areas, ventral striatum and cerebellum (Floyer-Lea & Matthews, 2005; Grafton, Hazeltine, & Ivry, 2002). Several models were developed that attempt to provide useful framework for interpreting this complex pattern of brain activation (Doyon, Ungerleider, Squire, & Schacter, 2002; Hikosaka, Nakamura, Sakai, & Nakahara, 2002; Miller & Cohen, 2001). Integrating some of the common features of these perspectives, it can be argued that fast learning requires interaction between distinct cortical and subcortical regions, where different cortico-subcortical circuits facilitate learning of different aspects of the task (e.g. spatial coordinates, motor coordinates) (Doyon et al., 2002; Hikosaka et al., 2002). Further then, involvement of prefrontal areas suggest that this stage of learning requires significant amount of attention and executive resources (Miller & Cohen, 2001).

### **Slow Motor Learning**

Slow learning occurs at the later stage of the motor learning process. The functional improvements in this stage are quantitatively smaller, and generally develop at a slower pace (Karni et al., 1998) than those apparent during fast motor learning. As in the case of fast learning, the time course and magnitude of the changes are task dependant e.g. violin play can be continuously improved over years, while the four component key press is generally mastered over multiple sessions over a few weeks. As the task is practiced over and over again, it becomes automatised, and there is less need for executive resources such as attention (Dayan & Cohen, 2011). Subsequently, slow learning requires less involvement of the prefrontal

regions, and performance is facilitated by more rostral regions, such as primary somatosensory cortex, primary motor cortex, dorsal striatum and cerebellum (Floyer-Lea & Matthews, 2005; Lehericy et al., 2005). Further then, there is a decrease in connectivity between premotor and ventral (associative) striatum, and increased connectivity between primary motor cortex, dorsal (sensorimotor) striatum and cerebellum (Floyer-Lea & Matthews, 2005; Lehericy et al., 2005). This suggests a shift from skill acquisition (association formation) to habitual (automatic) performance, behaviour that is more stable over time, and less susceptible to cognitive and other motor task interference (Dayan & Cohen, 2011; Shiffrin & Schneider, 1977; Yin et al., 2009).

### **Motor Learning Induced Plasticity**

Using skilled motor reaching training in rodents, it was found that increases in the accuracy of the task performance is associated with formation of new, and enlargement of existing dendritic spines in the early stage of learning (Xu et al., 2009). In the later stage of learning, after 10 days of training, the increase in accuracy develops at a slower rate, and is associated with large scale reorganisation of the cortical maps in associated primary motor cortex (Kleim et al., 2004). Findings similar to these are commonly reported in studies investigating the effects of skilled motor learning on neural plasticity in the primary motor cortex (Bury & Jones, 2002; Kleim et al., 2004; J. A. Kleim et al., 2002; Wang, Conner, Rickert, Tuszynski, & Jon, 2011; Xu et al., 2009) and are summarised in Table 1. Taken together, it can be concluded that skilled motor learning promotes structural changes in the areas associated with task performance, processes associated with use-dependant neural plasticity (Caroni, Donato, & Muller, 2012). Therefore, in the present study, skilled

reaching task is considered an appropriate paradigm to study the molecular correlates of use dependant neural plasticity in a rodent model.

Table 1

*Selected Studies Investigating Synaptic Plasticity in Primary Motor Cortex Following Motor Learning Task in Rodents*

Study	Species	Findings
Kleim et al., 2002	Rat	Significant increase in synapse per neuron in animals that were trained in reaching compare to untrained animals
Bury et al., 2002	Rat	Enhanced dendritic plasticity evidenced by increase in surface density of microtubule-associated protein 2 (MAP2) - cytoskeletal protein closely related to dendritic shape
Kleim et al., 2004	Rat	Increased number of synapses, and enlargement of cortical maps in later phase of skilled learning
Xu et al., 2009	Mouse	Motor learning induced rapid formation of dendritic spines on output pyramidal neurons in layer V. Spines were preferentially strengthen with subsequent training
Wang et al., 2011	Rat	Increase in dendritic branching and spine density in layer V pyramidal neurons. These changes were restricted to neurons projecting to C8 spinal cord segment controlling distal forelimb movement (e.g. digits for grasping)



## **Molecular Mechanisms of Use Dependant Plasticity**

Use dependant plasticity refers to reorganisation of nervous system in response to incoming neural stimulation produced by an individual's activity. There are several known physiological mechanisms of neuroplasticity that can be broadly categorised as processes related to neurogenesis, synaptic modifications, and myelination remodelling (Pascual-Leone et al., 2005). Synaptic changes, in particular, have received a lot of scientific attention, and are now widely considered to be the neural mechanism underpinning learning, memory and skills acquisition (Caroni et al., 2012; Malenka & Bear, 2004; Pascual-Leone et al., 2005). Neural synapses are specialised junction between neurons that allows for transmission of neural signals from the presynaptic membrane of terminal axon to postsynaptic membrane of dendritic spine (Caroni et al., 2012). Electrical impulses in the presynaptic neuron trigger the release of neurotransmitters at the terminal end of the axon, which act on the postsynaptic neuron, changing its membrane potential (Malenka & Bear, 2004). These changes of the postsynaptic membrane potential can either facilitate further propagation of the neural signal by excitatory transmission, or block further propagation by inhibitory transmission (Vitureira & Goda, 2013). The pattern and efficacy of synaptic activation are associated with long-term structural and functional changes at the synapse in form of strengthening (long-term potentiation [LTP]) or weakening (long-term depression [LTD]) of synaptic transmission (Malenka & Bear, 2004). At the presynaptic membrane, the efficacy of neural transmission is linked to the likelihood of neurotransmitter release, and at

postsynaptic membrane, it is linked to the number of receptors available to bind the released neurotransmitter (Vitureira & Goda, 2013).

### **Role of Synaptophysin in Presynaptic LTP**

One of the key presynaptic mechanisms facilitating LTP is the process of exo- and endocytosis of synaptic vesicles - a membrane-based organelles situated in the axon terminal that contain the neurotransmitters (Takamori et al., 2006). When action potential reaches axon terminal, it instigates the fusion of the synaptic vesicle with presynaptic membrane, and subsequent release of the neurotransmitters into the synaptic cleft (Sudhof, 2004). After the fusion, the synaptic vesicle is recycled back into to the cell, and refilled with neurotransmitters (Sudhof, 2004). One of the proteins facilitating this cyclic process of vesicular exo- and endocytosis is Synaptophysin. In particular, Synaptophysin was reported to be directly involved in biogenesis (Cameron, Sudhof, Jahn, & De Camilli, 1991), endocytosis (Daly, Sugimori, Moreira, Ziff, & Llinas, 2000; Kwon & Chapman, 2011), as well as exocytosis (Edelmann, Hanson, Chapman, & Jahn, 1995) of the synaptic vesicle. Functional importance of Synaptophysin has been studied using genetically manipulated animal strains with inactivated genes (knock-out models) that regulate the synthesis of Synaptophysin and its isoforms (Kwon & Chapman, 2011). It was found that Synaptogyrin/Synaptophysin double knock-out mice exhibited deficiency in establishing both, short and long-term potentiation (Janz et al., 1999). These results were more recently supported by findings of increased exploratory behaviours, compromised spatial learning, and reduced novelty object recognition in a Synaptophysin knock-out mice (Schmitt, Tanimoto, Seeliger, Schaeffel, & Leube, 2009). Overall, it can be concluded that by facilitating the process of

neurotransmitter release into synaptic cleft, Synaptophysin plays a crucial role in facilitation of LTP and associated learning (Janz et al., 1999; Schmitt et al., 2009).

## **Functional and Structural Changes at Postsynaptic Membrane During**

### **Induction of LTP**

Functional changes in synaptic strength are also accompanied by structural changes at the postsynaptic membrane. More specifically, it is suggested that LTP is linked to formation and/or enlargement of dendritic spines (protrusions of dendrites that typically receive neural signal) and axonal sprouting, while LTD is linked to spine shrinkage and dendritic pruning (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004; Zhou, Homma, & Poo, 2004). The process of enlargement/shrinkage of dendritic spine is linked to the presence/absence of receptors at the postsynaptic membrane. When excitatory neurotransmitter glutamate is released by presynaptic cell, it binds to N-methyl-D-aspartate receptors (NMDA-R), ligand-gated receptors of postsynaptic membrane. Activation of NMDA-R requires three events to co-occur a) binding glutamate, b) binding of antagonist glycine, and c) depolarisation of the postsynaptic membrane to a point when magnesium ( $Mg^{+}$ ) block on NMDA-R is removed (Furukawa, Singh, Mancusso, & Gouaux, 2005; Johnson & Ascher, 1987; MacDermott, Mayer, Westbrook, Smith, & Barker, 1986; Malenka & Bear, 2004). The co-occurrence of these three events leads to an opening of the NMDA-R, and a subsequent influx of Calcium ( $Ca^{2+}$ ) into the cell (Furukawa et al., 2005). The role of  $Ca^{2+}$  inside of the cell is numerous (Clapham, 2007), however, in respect to the LTP, it was demonstrated that influx of  $Ca^{2+}$  triggers a cascade of molecular events that result in a structural and functional changes facilitating the process of synaptic strengthening (Malenka & Bear, 2004). One of the key events is the activation of  $Ca^{2+}$ /calmodulin dependant kinase II, and protein kinase C, which results in an

increased phosphorylation and trafficking of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-R) into the postsynaptic membrane (Hanley, 2008; Malenka, Kauer, Zucker, & Nicoll, 1988). Just like NMDA-R, AMPA-R are a ligand-gated receptors of excitatory glutamate neurotransmitter, and facilitate fast synaptic transmissions (Furukawa et al., 2005). Increased number of AMPA-R in the postsynaptic membrane increases the likelihood of neural signal propagation, as well as it leads to an increase of the overall size of the dendritic spine (Huganir & Nicoll, 2013; Makino & Malinow, 2009). Thus, the insertion of AMPA-R into postsynaptic membrane leads to functional changes in form of increased activity at the synapse, as well as structural changes in form of enlargement of existing or generation of new dendritic spines (Hanley, 2008; Huganir & Nicoll, 2013; Makino & Malinow, 2009).

### **Role of PSD-95 in the Maintenance of Postsynaptic LTP**

Induction of synaptic LTP is followed by a complex set of cellular events that facilitate maintenance of morphological synaptic changes over time, however, precise mechanisms of these events are not fully understood yet (Meyer, Bonhoeffer, & Scheuss, 2014). One of the key events consistently reported to be involved in stabilisation of enlarged synaptic spine, is anchoring of inserted AMPA receptors at the cell membrane by proteins of postsynaptic density (Malinow & Malenka, 2002; Matsuzaki et al., 2004; Meyer et al., 2014; Murakoshi & Yasuda, 2012). Postsynaptic density is a protein rich subsynaptic structure that provides structural support to the receptors at the membrane, as well as ensures the alignment of these receptors with the sites of presynaptic neurotransmitter release (Meyer et al., 2014). PSD contains large and diverse set of proteins, and changes in protein composition of PSD control the anchoring of receptors in the postsynaptic membrane, thus, influence the strength

of the synapse (Vitureira & Goda, 2013) . The best studied protein of PSD is the postsynaptic density protein 95 (PSD-95), that acts as a scaffolding protein for both AMPA and NMDA receptors (Hunt, Schenker, & Kennedy, 1996). Optimal levels of PSD-95 are required for maintenance of activity induced synaptic strengthening (Ehrlich, Klein, Rumpel, & Malinow, 2007). Altered levels of PSD-95 were also linked to abnormal behaviour in animal knock-out models. For example, compared to a wild type mice, genetically manipulated mice with reduced expression of PSD-95 had impaired performance on a water maze task, suggesting deficits with spatial learning and memory formation (Migaud et al., 1998).

Taken together, it can be concluded that PSD-95 plays an important role in facilitating the maintenance of activity dependant LTP on the postsynaptic membrane, and is a crucial component in the process of learning and memory formation.

### **Role of Inhibition in Modulating Neural Activity**

The plasticity of inhibitory neural networks has received relatively less research attention than its excitatory counterpart, and therefore the role of interneurons in learning and memory formation is yet to be fully understood. One of the reasons why this area is relatively understudied is the diversity and complexity of the inhibitory effects (Markram et al., 2004). There are two main forms of inhibition modulating neuronal activity: feedforward (interneuron inhibits activity of cells other than the one from which it received neural input) and feedback (interneuron inhibits activity of the cell from which it received neural input). This type of signalling has been observed in a relatively well described class of interneurons expressing calcium-binding proteins Calretinin and Parvalbumin (Hu, Gan, & Jonas, 2014). Calcium-binding proteins are involved in modulation of calcium-signalling, event

closely related to changes in cell's excitability and plasticity (Berridge, 1998; Clapham, 2007). These proteins are found in interneurons expressing inhibitory GABA neurotransmitter, and generally, the cell only expresses one of the proteins, which makes them well suited for individual examination (Barinka & Druga, 2010).

### **Calretinin and Parvalbumin.**

Parvalbumin positive ( $PV^{+}$ ) interneurons are mainly involved in feedforward inhibition, and synapse on both soma and axon of the principal excitatory neurons (Hu et al., 2014). Like all calcium-binding proteins,  $PV^{+}$  cells are involved in regulation of intracellular calcium-signalling of the neuron they synapses onto (Caillard et al., 2000).  $PV^{+}$  interneurons are fast conducting cells that have their output sites tightly aligned with calcium channels of the cell receiving their inhibitory output (Hu et al., 2014). This combination of fast signal propagation, and proximity to the site of influence renders  $PV^{+}$  interneurons a very effective modulator of neural activity (Chen, Kim, Peters, & Komiyama, 2015). Studies concerned with functional importance of Parvalbumin suggest its involvement in learning and memory formation. For example, the stimulation of  $PV^{+}$  interneurons in primary motor cortex was linked to acceleration of extinction of reward seeking behaviour (Sparta et al., 2014), and PV induced inhibition was found to facilitate auditory fear conditioning response (Wolff et al., 2014).

Calretinin positive ( $CR^{+}$ ) cells are predominantly located in the superficial cortical layers II and III (Barinka & Druga, 2010). Using *in vitro* cellular models, it was found that Calretinin is abundantly present in both presynaptic and postsynaptic neuritis of  $CR^{+}$  cells, and regulates both amplitude and duration of calcium-signalling (Barinka & Druga, 2010; Edmonds, Reyes, Schwaller, & Roberts, 2000; Faas, Schwaller, Vergara, & Mody, 2007). The importance of Calretinin in learning

and memory formation was demonstrated using Calretinin knock-out models. Animals with decreased expression of Calretinin exhibited reduced capacity for LTP (Gurden et al., 1998), abnormal cortical excitability, and impairment of motor coordination (Gall et al., 2003).

Overall, it can be concluded that calcium-binding proteins Parvalbumin and Calretinin are important modulators of neural excitability. By regulating calcium-signalling of connecting cell, they are directly involved in regulation of synaptic plasticity (Clapham, 2007). Further then, the non-overlapping expression of these proteins within the cell makes them a well suited target for individual examination.

### **Passive Induction of Plasticity Using rTMS**

Neuroplastic changes resembling those associated with activity dependant plasticity in motor learning have been observed in humans and rodents following passive brain stimulation using rTMS (Cramer et al., 2011). During rTMS session, an electromagnetic coil is used to generate a transient magnetic field that can painlessly penetrate the skull, and – depending on frequency of the stimulation - either increase ( $\geq 5$  Hz) or decrease ( $\leq 1$  Hz) the neural activity (Funke & Benali, 2011). Using a particular rTMS protocol of theta burst stimulation (TBS), it was found that the effects of stimulation can outlast the duration of the stimulation (Huang, Edwards, Rounis, Bhatia, & Rothwell, 2005). TBS consists of bursts of theta pulses generated at equally spaced intervals, and when the trains of theta pulses are delivered in a continuous manner (cTBS) the cortical activity decreases, while intermittent delivery (iTBS) can increase the cortical activity (Huang et al., 2005)

## **Effects of rTMS on Motor Learning**

The effects of various rTMS protocols on motor performance have been extensively studied over the last decade in both humans and animals. In human research, it has been demonstrated that delivery of rTMS directly prior to motor learning can enhance the encoding of motor memory (Bütefisch, Khurana, Kopylev, & Cohen, 2004), improve consolidation of learned motor skill (Boyd & Linsdell, 2009) and increase accuracy of motor sequence task performance (Narayana et al., 2014). The facilitatory effects of iTBS protocols were investigated by pairing iTBS with various motor learning tasks. It was found that when delivered immediately before the training, iTBS can enhance the effects of motor learning (Teo, Swayne, Cheeran, Greenwood, & Rothwell, 2011), facilitate movement preparation (Stinear et al., 2009), improve outcomes of a grip-lift exercise in stroke patients (Ackerley, Stinear, Barber, & Byblow, 2014), enhanced the rate of skill acquisition in a sequential motor task (Narayana et al., 2014). Similarly, in animals iTBS was found to increase accuracy on skilled reaching task (Tang, Bennett, et al., 2015), as well as increase performance on associative tactile learning in rats (Mix, Benali, Eysel, & Funke, 2010).

## **Molecular Changes in Response to rTMS**

Neurophysiological changes involved in use-dependant synaptic modulation were also observed following rTMS stimulation, (Funke & Benali, 2011; Ma et al., 2013; A. Mix, Benali, & Funke, 2014; Vlachos et al., 2012), suggesting that changes in synaptic transmission could be a possible mechanism by which rTMS protocols induce lasting behavioural changes. However, these findings are often contradictory. Vlachos et al. (2012) reported LTP-like enlargement of dendritic spines on excitatory pyramidal neurons of *in vitro* cultured hippocampal slices following high-frequency



rTMS ( $\geq 5$  Hz). These findings were replicated by Lenz et al. (2014), who also reported that the enlargement was induced preferentially on dendrites proximal to the cell body. Investigating the effects of high-frequency rTMS on inhibitory circuitry, Funke and Benali (2011) found downregulation of calcium binding protein Parvalbumin in the inhibitory interneurons, event that could potentially lead to facilitation of excitation in the excitatory neurons receiving signal from these interneurons. Contrary to these findings, Ma et al. (2013) reported increase in LTP associated molecular markers Synaptophysin and PSD-95 following inhibitory rTMS ( $\leq 1$  Hz) in aged mice, findings that were recently replicated by Zhang et al. (2015).

Reasons for differential findings of these studies could be various. There were methodological differences with respect to the stimulation protocols, as well as differences in the animal models used (*in vitro*, aged vs. young rodents). In addition, in all of the above mentioned studies, a human sized coil was used on rodents, potentially compromising the translational merit of these studies (stimulation in humans is delivered focally, and penetrating only the superficial layers of cortex).

Table 2

*Key Studies Examining Effects of rTMS on Markers of Plasticity in Excitatory, and Inhibitory Cells*

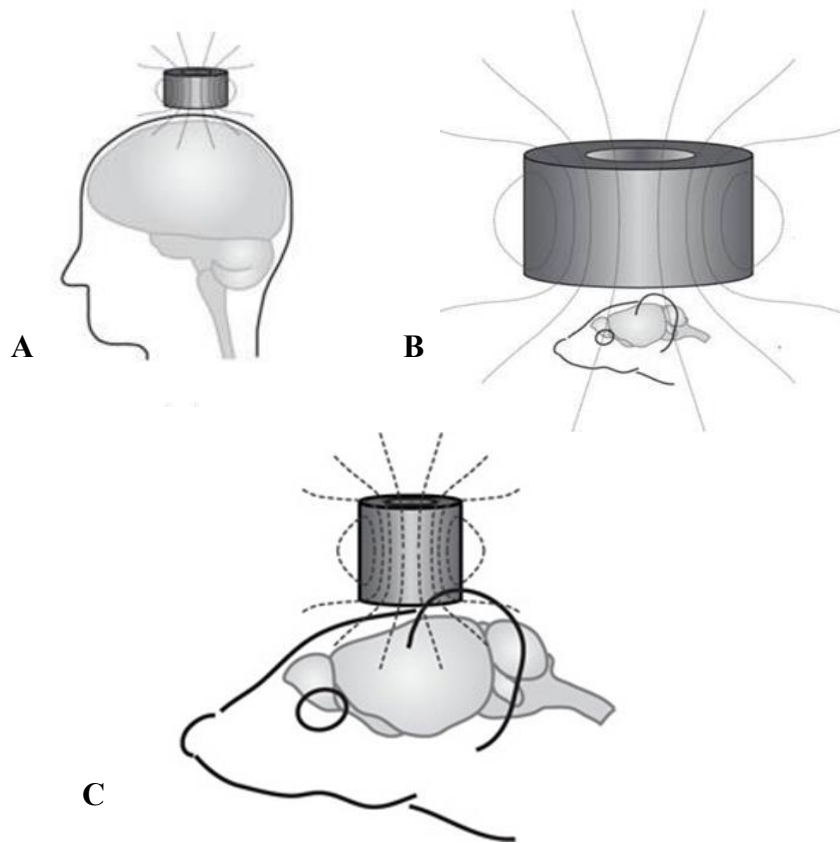
Study	Cell type	rTMS	Findings
Vlachos et al. (2012)	Excitatory	Excitatory	Upregulation of postsynaptic markers F-actin, and GluA1. No change in in presynaptic Synaptophysin
Ma et al. (2014)	Excitatory	Excitatory/Inhibitory	Synaptophysin, GAP43, PSD-95 were upregulated following inhibitory, and downregulated following excitatory stimulation
Zhang et al. (2015)	Excitatory	Inhibitory	Upregulation of synaptophysin, GAP43
Lee, Oh, Kim, and Paik (2014)	Excitatory	Excitatory	Upregulation of Synaptophysin following single stimulation, no change following 20 sessions
Mix et al. (2010)	Inhibitory	Excitatory	Reduced expression of parvalbumin and GAD67
Benali et al. (2011)	Inhibitory	Excitatory/Inhibitory	Reduced expression of parvalbumin following excitatory stimulation, and reduced expression of calbindin following inhibitory stimulation

### **Purpose of this study**

Investigation of the effects of rTMS at the behavioural and systemic level suggest that these are facilitated by LTP/LTD-like changes commonly associated with use-dependant plasticity. However, the investigation of molecular markers of the suspected LTP/LTD events is currently inconclusive, and methodologically somewhat distanced from the human condition due to the use of oversized human coils in the experimental animal models.

Therefore, the present study aims to investigate whether the effects of rTMS are facilitated by known LTP/LTD like events at the cellular level while maintaining high translational merits of these findings to the human condition. The investigation is undertaken by means of immunochemical analysis, well-established technique for detection of wide range of tissue constituents specific to particular cell subpopulation (De Matos, Trufelli, de Matos, & da Silva Pinhal, 2010).

Present study is conducted within a larger multidisciplinary NHMRC approved project (CIA Prof Jeffery Summers) that investigates physiological effects of rTMS through translational research, linking the evidence obtained from animal studies to human condition. Within this project, high translational merits of findings obtained from animal experiments are achieved by using a well-established skilled forelimb reaching motor learning in rodents, a paradigm equivalent to skilled manual dexterity learning in humans (Whishaw et al., 1992). In addition, all animal stimulation is performed using a custom-build rodent-scaled coil (Figure 1), developed and validated by Tang, Garrett, et al. (2015).

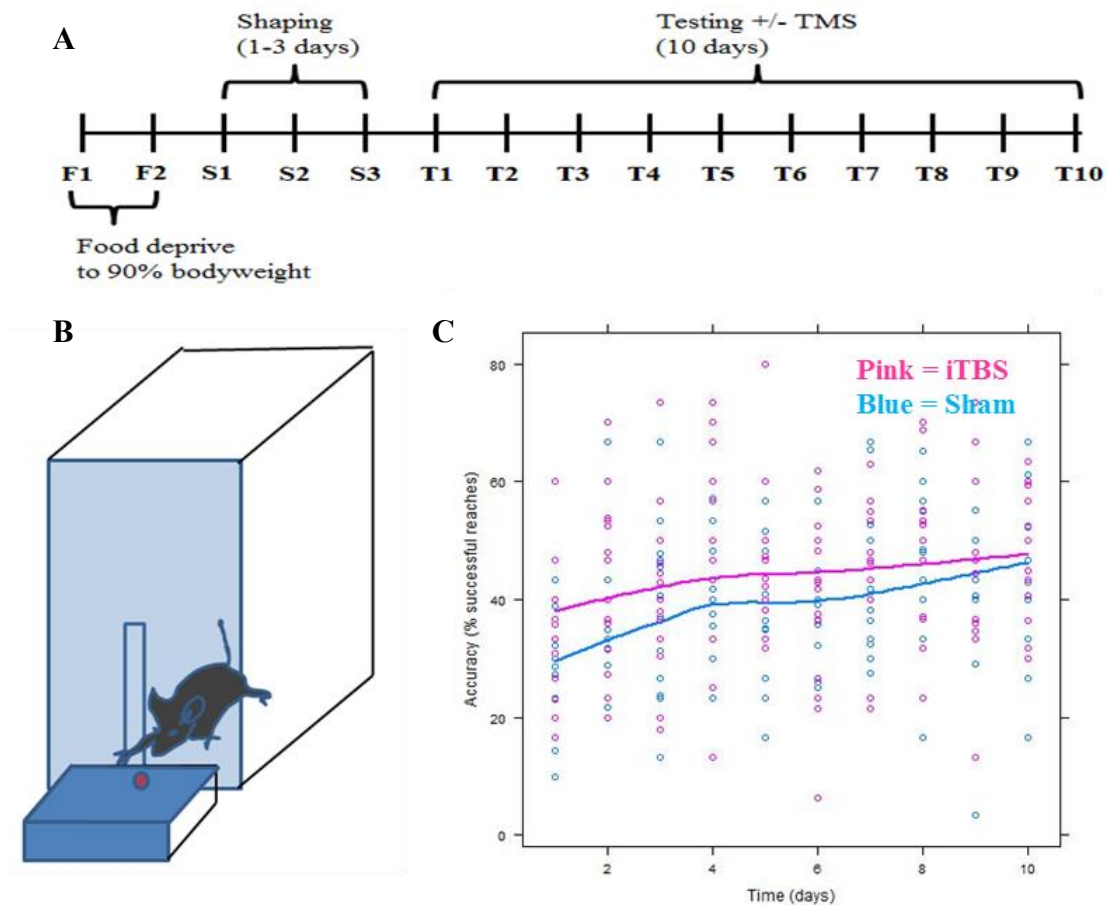


*Figure 1*

Use of species specific electromagnetic coil in humans and rodents. **A** Focal stimulation in humans **B** Magnetic field generated by a human sized coil affects the entire rodent brain **C** Rodent-specific coil has a focal effect comparable to that used in humans (Tang, Bennett, et al., 2015).

Previous findings obtained within this project suggest that iTBS stimulation delivered prior to skilled reaching in rodents has the capacity to facilitate performance on this task (Tang, Bennett, et al., 2015). In particular, a significant increase in accuracy was found over a 10 days of training in the group that received iTBS over primary motor cortex contralateral to the dominant paw prior to motor training, compared to the control group that did not receive the stimulation (Tang, Bennett, et al., 2015) (see Figure2). This finding of performance facilitation

following iTBS is consistent with those reported in human skilled manual dexterity studies (e.g. Narayana et al., 2014).



*Figure 2*

Skilled motor reaching. **A** Timeline of the behaviours experiments in days. For motivation to participate, all animals were food deprived to 90% of body weight over 2 days (F1-F2), the paw preference was established over 3 days (S1-S3), followed by 10 days of training (T1-T10). **B** Skilled reaching training using dominant paw; animals had to retrieve food pellet through slit in a perplex glass box **C** Animals that received iTBS prior training (pink line) outperformed those that did not (blue line). X axis indicates time, Y axis indicates accuracy as % of successful reaches (Tang, Bennett, et al., 2015).

At the conclusion of behavioural experiments, the animals in Tang, Bennett, et al. (2015) study were transcardially perfused, the brain tissue was harvested and stored for further molecular analyses. In addition to the two experimental cohorts (iTBS, SHAM), a cohort that was matched for handling only was generated (HAN; animals were taken through the same day/night routine as the experimental cohort, but without any experimental manipulation). Brain tissue from the handling cohort was harvested and stored in the same fashion as the tissue from the experimental cohort. The aim of the current project was to perform molecular analysis by means of immunohistochemistry on the brain tissue obtained from these three animal groups (iTBS, SHAM, HAN), and investigate quantitative differences in expression of known neurochemical markers of plasticity between these groups within relevant brain areas of hemisphere contralateral to the dominant paw. Previously, it has been established that 10 days of skilled motor learning in rodents involves slow-motor learning (Kleim et al., 2004), therefore, the brain areas selected for investigation in this study were: a) primary motor cortex that provides output to muscles of the dominant paw (Xu et al., 2009) b) dorsal striatum associated with slow motor learning (Kleim et al., 2004); and c) the piriform cortex associated with processing of olfactory information (Watson, Kirkcaldie, & Paxinos, 2010) which served as a control area not associated with motor learning, and not exposed to the stimulation.

### **Aims and Hypotheses**

The cumulative evidence of enhancement in performance (Narayana et al., 2014; Tang, Bennett, et al., 2015), increase of cortical excitability (Huang et al., 2005), dendritic enlargement (Lenz et al., 2014; Vlachos et al., 2012) and decrease in the expression of inhibitory markers (Funke & Benali, 2011) observed following

high frequency rTMS, suggest the presence of underlying LTP-like neural mechanisms. Therefore, given that animals in Tang, Bennett, et al. (2015) received high frequency iTBS, a significant increase in the expression of excitatory proteins PSD95 and Synaptophysin, and significant decrease in inhibitory proteins Parvalbumin and Calretinin was hypothesised in the primary motor cortex and dorsal striatum of hemisphere contralateral to the dominant paw, in the group that received iTBS, compared to both SHAM and HAN groups. No significant difference in expression of these markers was hypothesised in the piriform cortex.

To test these hypotheses, the project was divided into two stages. In the first stage, the immunofluorescent protocols for the markers of interest were optimised in non-experimental tissue obtained from the same strain of mice. In the second stage, the experimental tissue samples were probed using the optimised protocols and the abundance of labelled biomarkers was quantified by means of confocal microscopy and subsequent image analysis.

## **Method**

### **Subjects**

The samples consisted of brain tissue obtained from twelve (12) genetically identical C57B16 mice, however, four (4) had to be excluded from the analysis due to inability of the antibody to penetrate the cells and bind the antigen of the interest. Thus, the final analysis was performed on brain tissue of eight ( $N=8$ ) animals from three randomly assigned experimental groups: those that received iTBS prior to learning skilled reaching (iTBS,  $n=2$ ), those that received sham stimulation prior to learning (SHAM,  $n=3$ ), and group that was matched for handling only (HAN,  $n=3$ ). All mice were group housed males that were 10-20 weeks old at the commencement

of the experiments. In order to ensure that the animals were sufficiently motivated to work for a food reward, they were food deprived to 90% of body weight throughout the whole experimental period. Access to water was unrestricted.

## **Ethics**

All experimental procedures were approved by the Animal Ethics Committee of the University of Tasmania A13168, and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

## **Materials and Procedures**

### **Tissue preparation and cryosectioning.**

Upon conclusion of the behavioural experiments, a standard fixation procedure was performed (4 % paraformaldehyde [PFA] perfused via the vascular system through the heart), the brains were harvested, postfixed in 4 % PFA for 24 hours, and stored in a standard tissue storage buffer of Phosphate-Buffered Saline (PBS) with 0.2 % Sodium Azide (preservative inhibiting potential bacterial growth). Prior to cryo-sectioning, the brains were removed from PBS 0.2 % Sodium Azide, and cryo-protected to prevent ice crystal formation and associated tissue damage. This was achieved by submerging the brain tissue in 18 % and 30 % sucrose successively, allowing sucrose to replace the water content in the cells. Sinking of the tissue to the bottom of the vial was considered indicative of a successful sucrose infiltration. Following this, the samples were mounted into plastic moulds using an optimal cutting temperature [OCT] compound medium, and stored at -20 °C. 40 µm coronal sections were obtained using Cryostat cooled to -18°C, and the obtained sections were then stored at 4 °C in PBS 0.2 % Sodium Azide buffer.



### **Optimisation of immunofluorescent labelling.**

Several rounds of immunofluorescent labelling were performed on non-experimental tissue to develop an optimal labelling protocol. Two variables were manipulated during the optimisations, a) concentration of the primary antibody, and b) blocking agent used for reduction of the non-specific binding (10 % horse serum, Dako<sup>®</sup> serum free blocking agent). Each round of optimisation also included a no primary and no secondary antibody control condition, for determination of the overall auto-fluorescence of the tissue in the absence of each antibody. The final protocol consisted of washing the brain sections with PBS 0.6% Triton X three times for 10 minutes, incubating the sections for 30 minutes in Dako<sup>®</sup> serum free blocking agent to prevent non-specific binding of the antibody. This was followed by an overnight incubation of primary antibodies at 4 °C on a shaker, to ensure complete penetration of the antibody to the tissue (see Table 2 for details of primary antibodies). The next day the sections were washed with PBS 0.6% Triton X (three times 10 minutes on shaker), and a secondary fluorescent antibody (Alexa Fluor<sup>®</sup> 488, 1:1000) was incubated for two hours at room temperature on the shaker. After this two hour incubation, the sections were finally washed in PBS (three times 10 minutes on shaker). Following, all sections were mounted onto FLEX IHC Microscope Slides using Fluorescence Mounting Medium and 24x55 mm Cover Glass coverslips (all Dako<sup>®</sup>), and left overnight at a room temperature which allowed the mounting medium to set.

Table 3

*Primary Antibodies Used for Immunofluorescent Labelling*

Antigen	Type	Species of host	Manufacturer	Concentration
Calretinin	Polyclonal	Rabbit	Swant	1:2000
Parvalbumin	Monoclonal	Mouse	Swant	1:1000
Synaptophysin	Polyclonal	Rabbit	Milipore	1:200
PSD-95	Monoclonal	Mouse	Abcam	1:500

**Experimental tissue treatment.**

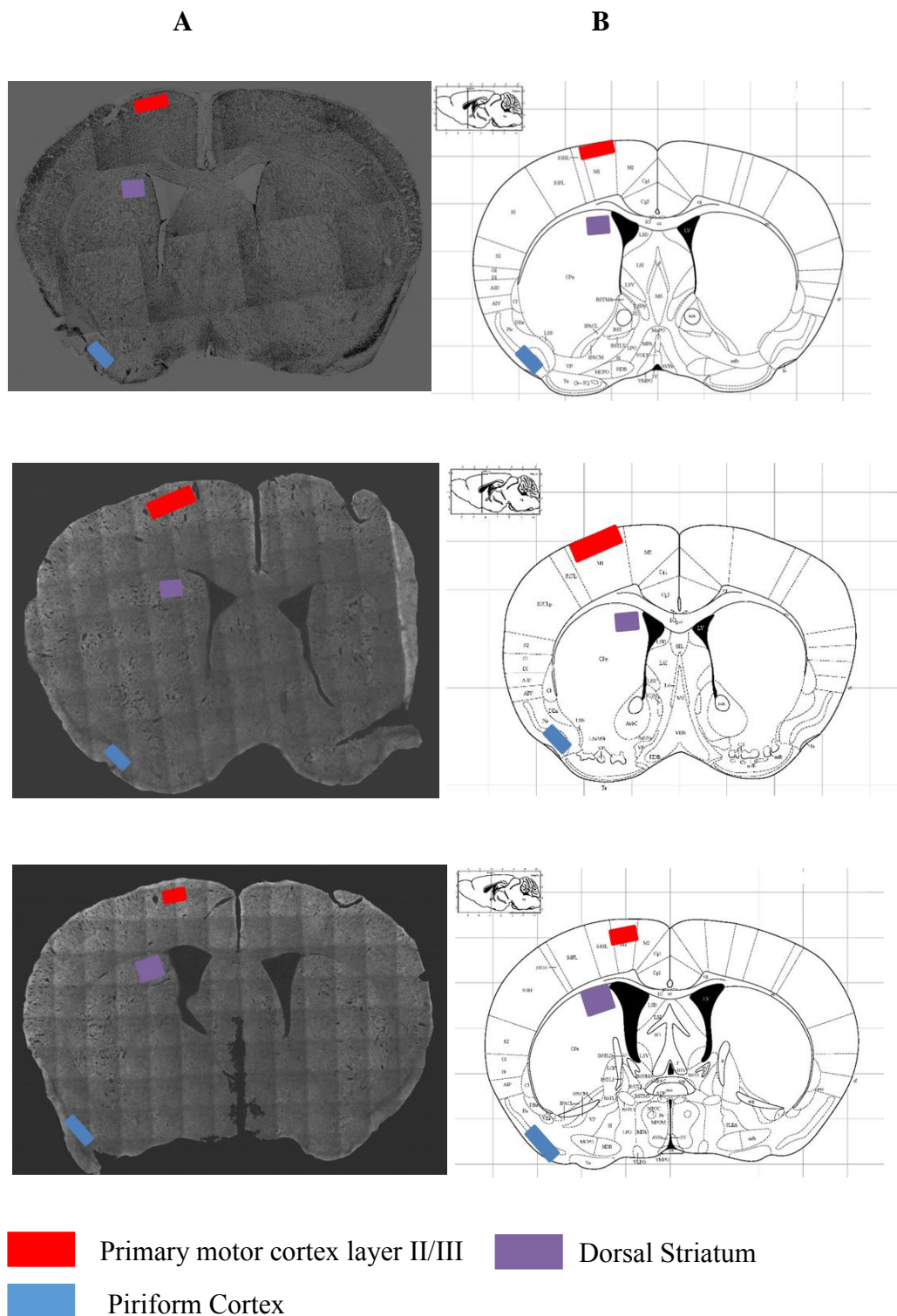
Despite the optimisation of procedures in the non-experimental tissue, it eventuated that the experimental tissue required further treatment, as the optimised protocol did not translate successfully. The binding of antibody across different sections was inconsistent, with some sections failing to produce labelling altogether. Commonly, the binding of antibody to an antigen is influenced by the effects of PFA based fixatives (such as 4% PFA used in the current study). Prolonged PFA exposure can change the three-dimensional structure of the proteins due to cross-linking of antigens and unrelated tissue proteins, which can render the targeted antigens undetectable for the antibody (Ramos-Vara & Miller, 2014). To reverse this effect and increase the overall immunoreactivity, the experimental tissue was subjected to heat-induced epitope retrieval. Sections were submerged into a pH adjusted (pH = 6.0) citrate buffer solution, and placed in a 90 °C water bath for 20 minutes (see

Appendix A for full protocol). After cooling back to the room temperature, the sections were immunofluorescently labelled as per optimised protocol (see previous section ‘Optimisation of Immunofluorescent Labelling’). Alternative treatment of autofluorescence quenching was aimed at reducing the autofluorescence of the tissue caused by PFA reacting with amines and proteins within the tissue (Ramos-Vara & Miller, 2014). This was achieved by taking the sections through potassium permanganate and potassium metabisulfite washes (see Appendix A).

These procedures, however, did not improve the immunofluorescent labelling for any of the markers of interest. Therefore, all initial labelling (conducted prior tissue treatment procedures) of experimental tissue was reviewed. The best outcome was found in sections obtained from eight out of 12 animals (3 HAN, 3 SHAM, 2 iTBS), that were labelled with Synaptophysin. These eight Synaptophysin labelled experimental samples were used for subsequent quantification analysis.

### **Mouse brain atlas generation.**

To aid the identification of areas of interest in obtained coronal sections, one set of sections was stained using cresyl violet (see Appendix A). The sections were mounted onto Dako<sup>®</sup> microscopy slides, covered with cresyl violet for 20-30min, and then taken through series of washes from absolute alcohol into xylene, and Dako<sup>®</sup> glass cover slips were mounted over the sections using Pyrex mounting medium. Cresyl violet produces a Nissl-type stain marking cell nuclei, allowing for visualisation of major neuroanatomical landmarks. The stained sections were then visualised using x10 lens of Zeiss Confocal Spinning Disk Microscope. Captured images were then compared to the known neuroanatomical landmarks using Paxinos and Watson (2005) stereotaxic coordinates (see Figure 3).



*Figure 3*

Identification of areas of interest. **A** Coronal sections from C57B16 mice strain stained with cresyl violet **B** Known stereotaxic coordinates (Paxinos & Watson, 2005).

### **Microscopy and image analysis.**

For each area of interest, 4  $\mu\text{m}$  deep stitched images were obtained with x60 water lens using Zeiss Confocal Spinning Disk Microscope. These images were then transformed into a binary black and white representations using a custom segmentation plugin (O'Mara et al., in preparation) for the ImageJ software package (Figure 4). An area of the interest was defined for each image by creating a mask image in Photoshop<sup>®</sup> software, discounting the cell bodies and blood vessels. This mask was then overlayed over the segmented image using ImageJ software. The antibody labelling was then quantified and measured within the pre-defined area of interest using the analyse particle function in ImageJ software package (Schindelin, Rueden, Hiner, & Eliceiri, 2015).

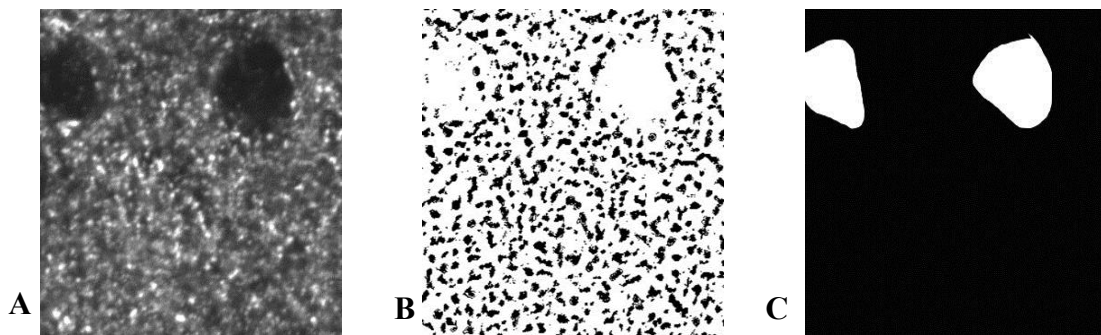


Figure 4

Process of image analysis. **A** Original image obtained from the confocal microscope **B** Segmented binary image **C** Mask created in Photoshop<sup>®</sup>, black area depicts the neuropil of interest, and white area depicts areas that were excluded from the analysis (cell bodies, blood vessels).

### **Design and data analysis.**

Differences in the levels of Synaptophysin expression were compared across the three experimental groups (iTBS, SHAM, HAN), for each area of the interest (primary motor cortex, dorsal striatum and piriform cortex). In particular, three measures were obtained: the number of synaptic puncta normalised per  $0.01\text{mm}^2$ ; the size of individual puncta in  $\mu\text{m}^2$ ; and the percentage of neuropil area covered in synaptic puncta.

It was intended to analyse the obtained data by Multivariate Analysis of Variance (MANOVA), with significant multivariate effects followed up by series of tests of simple main effects with Bonferroni adjusted  $\alpha$  level. If violation of assumption of homogeneity was suspected, the post-hoc analyses would have been interpreted using Games-Howell procedure, and if violation of multivariate homogeneity was suspected, Pillai's criterion would have been used to interpret the multivariate test. However, due to insufficient sample size (iTBS  $n=2$ , SHAM  $n=3$ , HAN  $n=3$ ), the analysis could not be performed. Instead, all the findings were described in terms of descriptive statistics, and 95% confidence intervals around the means were used to interpret statistical significance of the differences in scores for each measure separately.

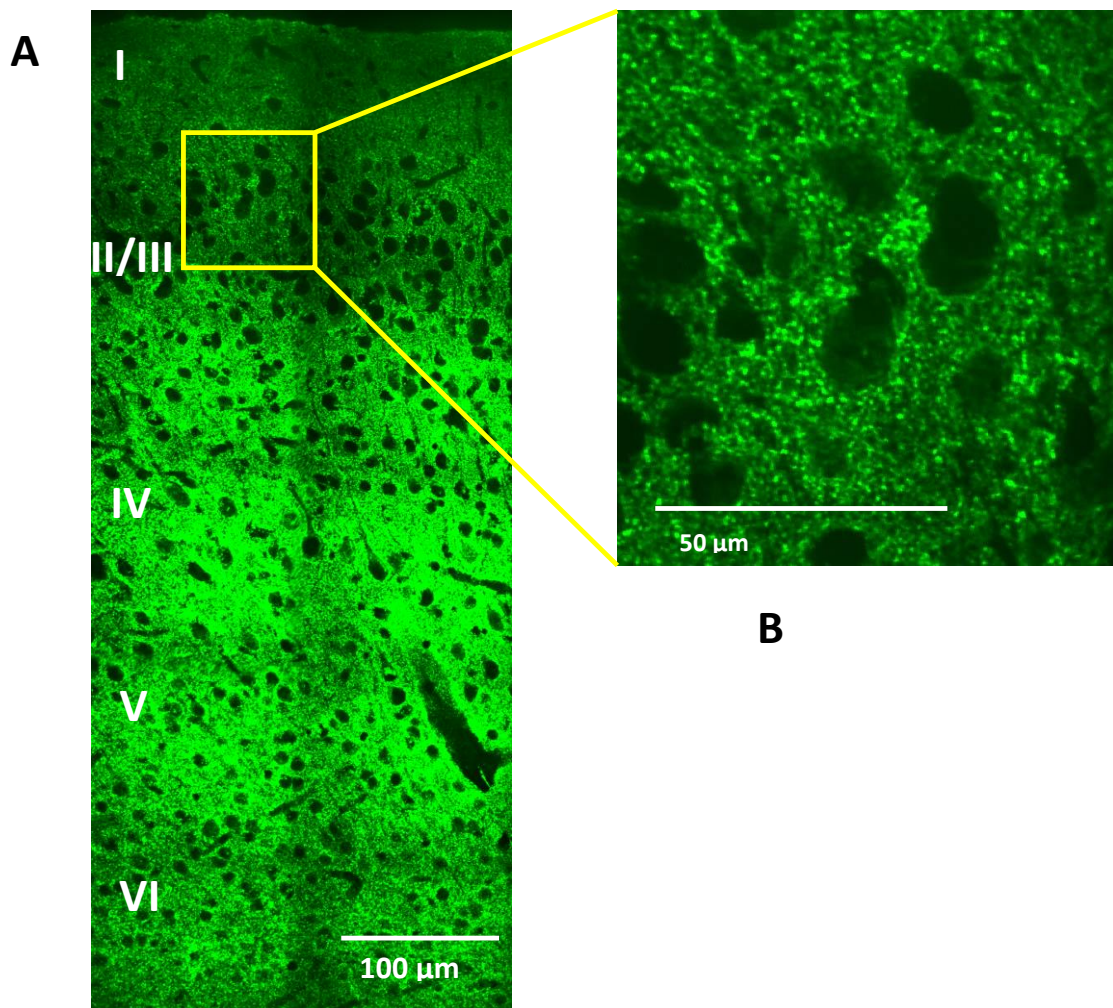
## **Results**

### **Aim 1- Immunofluorescent Labelling Optimised in Non-experimental tissue**

#### **Synaptophysin.**

The anti-Synaptophysin antibody produced expected pattern of labelling, marking active presynaptic sites of the excitatory neurons in a dense, puncta like

fashion (Figure 5). Due to the diminutive nature of the presynaptic site, the best imaging results were achieved using the high magnification x60 lens of the Zeiss Confocal Spinning Disk Microscope. By applying these methods, the labelling was visualised as individual synaptic puncta, suitable for quantification in terms of the size of each individual puncta in  $\mu\text{m}^2$ , the number of the puncta per  $0.01\text{mm}^2$  of the neuropil, and the percentage of the neuropil covered in the puncta.



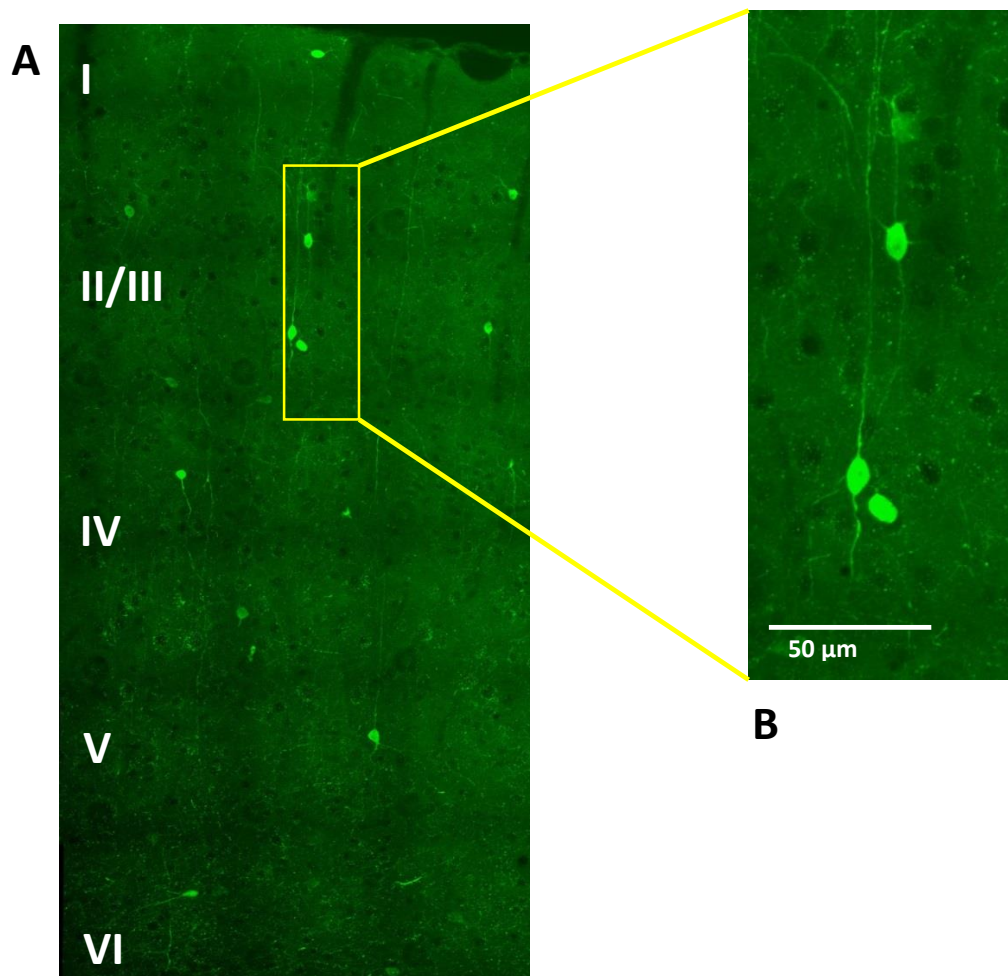
*Figure 5*

Pattern of Synaptophysin expression visualised using immunofluorescent labelling and confocal microscopy. **A** Cortical cross-section of primary motor cortex spanning from layer I to VI **B** Labelling pattern visualising sites of presynaptic activity.



### Calretinin.

Anti-Calretinin antibody visualised cells' somas and axons of inhibitory interneurons (Figure 6). The best imaging results were obtained with the x40 lens of the Zeiss Confocal Spinning Disk Microscope. Obtaining 10  $\mu\text{m}$  deep stacks of images would allow for tracing of the axon, and compare any morphological changes between the three treatment groups using Neurolucida<sup>®</sup> software package.



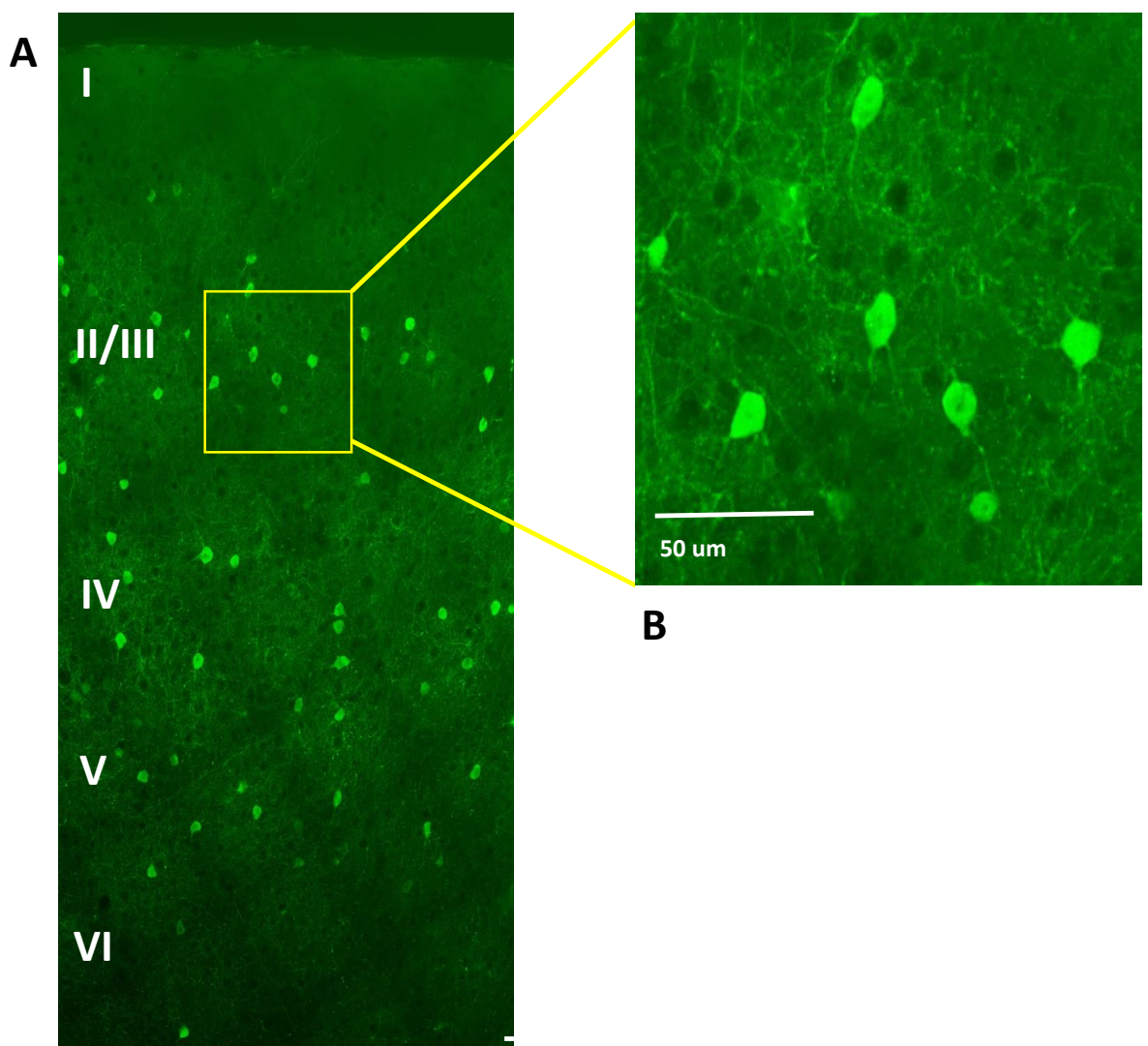
*Figure 6*

Pattern of Calretinin expression visualised using immunofluorescent labelling and confocal microscopy. **A** Cortical cross-section of primary motor cortex spanning from layer I to VI **B** Labelling pattern visualising cells' somas and axons.



### **Parvalbumin.**

Anti-Parvalbumin antibody produced a prominent labelling of soma, dendrites and axons, visualising overall morphology of the inhibitory interneurons (Figure 7). The best imaging results were obtained with x40 lens of Zeiss Confocal Spinning Disk Microscope. Visualised arborisation was too dense for individual tracing, therefore, the optimal method for quantification of this marker would have been the estimate of the percentage of the area covered by the labelled cells.



*Figure 7*

Pattern of Parvalbumin expression visualised using immunofluorescent labelling and confocal microscopy. **A** Cortical cross-section of primary motor cortex spanning from layer I to VI **B** Labelling pattern visualising cell morphology.

### **PSD-95.**

Anti-PSD-95 antibody obtained for the purpose of the labelling has unexpectedly failed to produce any results (see manufacturer statement in ‘General Notes’ section of the Appendix B). Typically, PSD-95 labelling appears as individual puncta along the axonal projections, and this type of labelling pattern could be quantified in terms of the number and the size of puncta within the area of interest as per Synaptophysin.

## **Aim 2 - Quantification of Synaptophysin Labelling in the Experimental Tissue**

### **Primary motor cortex.**

The average number of synaptic puncta per  $0.01\text{mm}^2$  was lowest for iTBS group, followed by HAN and SHAM groups. The average size of synaptic puncta was highest in the iTBS group, SHAM and HAN groups, and the average percentage of neuropil covered in synaptic puncta was highest in the iTBS group, followed by SHAM and HAN groups. Inspection of plots for 95% CI around means suggested that none of the observed differences in size, number or area covered by synaptic puncta between the three experimental groups were statistically significant (Table 4, Figure 8).

Table 4

*Means and Standard Deviation for Measures Obtained in Primary Motor Cortex*

	Number of puncta		Size of puncta		Area covered	
	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>
iTBS	50.62	1.23	0.55	0.28	19.50	7.77
SHAM	55.06	2.37	0.51	0.18	18.33	5.50
HAN	54.10	2.33	0.46	0.09	17.67	2.08

*Note.* *M* = Mean; *SD* = Standard Deviation; Number of puncta is calculated per 0.01mm<sup>2</sup> of labelled neuropil; Size of Puncta is in  $\mu\text{m}^2$ ; Area covered is in percentage

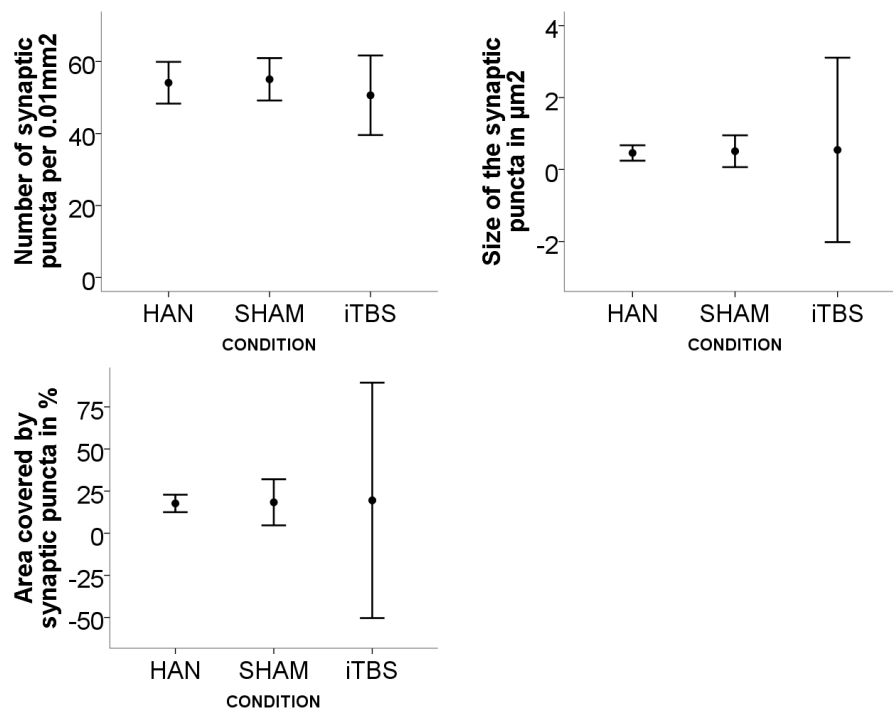


Figure 8

Graphs depicting 95 % confidence intervals around means for measures obtained in primary motor cortex. Number of puncta is calculated per 0.01mm<sup>2</sup> of the labelled neuropil.

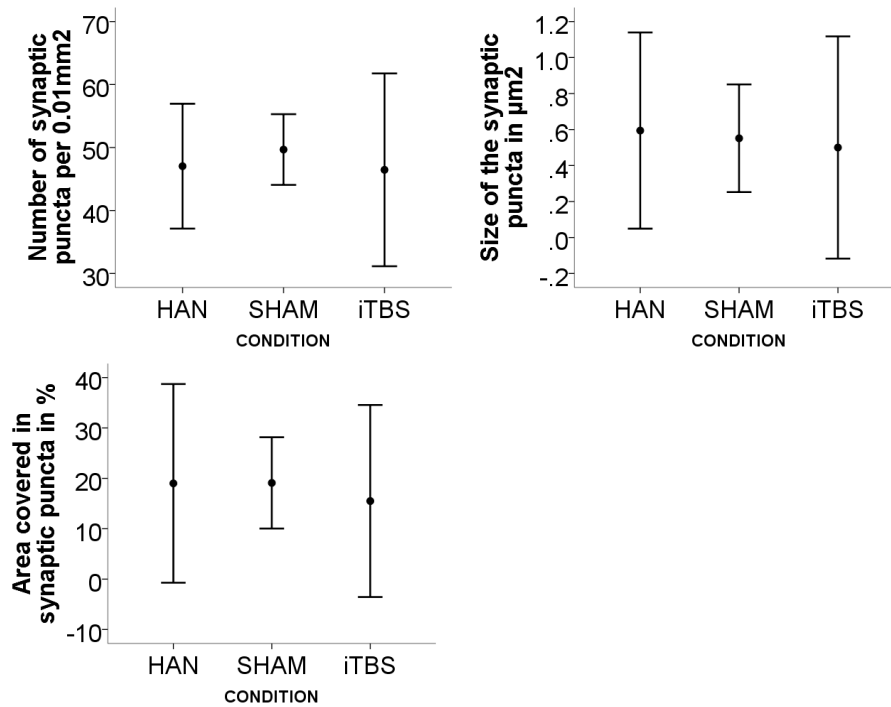
### Dorsal striatum.

The average number of synaptic puncta per 0.01mm<sup>2</sup> was lowest for the iTBS, followed by HAN and SHAM groups. The average size of synaptic puncta was lowest for the iTBS group, followed by SHAM and HAN groups, and the average percentage of neuropil covered in synaptic puncta was lowest in the iTBS group, followed by the HAN and SHAM. Inspection of plots for 95%CI around means suggested that none of the observed differences in size, number or area covered by puncta between the three experimental groups were statistically significant (Table 5, Figure 9).

Table 5  
*Means and Standard Deviation for Measures of Synaptic Puncta in Dorsal Striatum*

	Number of puncta		Size of puncta		Area covered	
	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>
iTBS	46.46	1.70	0.50	0.07	15.50	2.12
SHAM	49.67	2.25	0.55	0.12	19.09	3.65
HAN	47.04	3.99	0.59	0.21	19.00	7.94

*Note.* *M* = Mean; *SD* = Standard Deviation; Number of puncta is calculated per 0.01mm<sup>2</sup> of labelled neuropil; Size of Puncta is in µm<sup>2</sup>; Area covered is in percentage



*Figure 9*

Graph depicting 95 % confidence intervals around means for measures obtained in dorsal striatum. Number of puncta is calculated per 0.01mm<sup>2</sup> of the labelled neuropil.

### **Piriform cortex.**

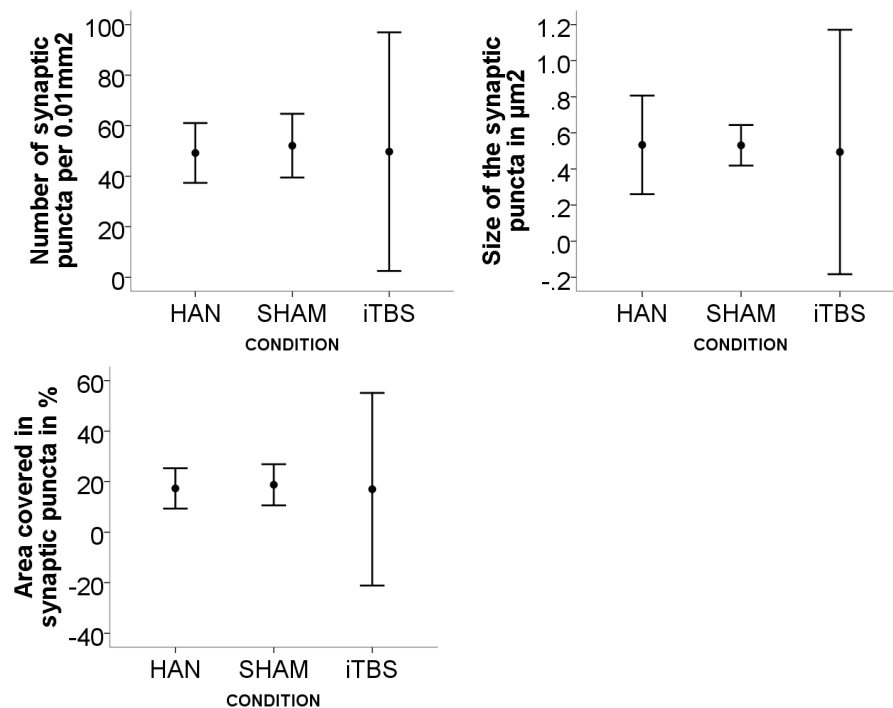
The average number of synaptic puncta per 0.01mm<sup>2</sup> was highest for the SHAM group, followed by iTBS and HAN groups. The average size of synaptic puncta was lowest in the iTBS group, followed by SHAM and HAN group, and the average percentage of neuropil covered in synaptic puncta was lowest in the iTBS group, followed by the HAN and SHAM groups. Inspection of plots for 95%CI around means suggested that none of the observed differences in size, number or area covered by puncta between the three experimental groups were statistically significant (Table 6, Figure 10).

Table 6

*Means and Standard Deviation for Measures of Synaptic Puncta in Piriform Cortex*

	Number of puncta		Size of puncta		Area covered	
	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>	<i>M</i>	<i>SD</i>
iTBS	49.71	5.26	0.49	0.07	17.33	3.21
SHAM	52.08	5.07	0.531	0.04	18.76	3.27
HAN	49.19	4.76	0.533	0.10	17.00	4.24

*Note.* *M* = Mean; *SD* = Standard Deviation; Number of puncta is calculated per 0.01mm<sup>2</sup> of labelled neuropil; Size of Puncta is in  $\mu\text{m}^2$ ; Area covered is in percentage



*Figure 10*

Graph depicting 95 % confidence intervals around means for measures obtained in piriform cortex. Number of puncta is calculated per 0.01mm<sup>2</sup> of the labelled neuropil.

## **Power Analysis and Sample Size**

Due to above mentioned paraformaldehyde induced tissue degradation, four experimental samples had to be excluded from the analysis. To define the optimal number of animals for any future investigation, a G\*Power analysis was performed. If significant multivariate effect existed, to detect this with MANOVA ( $\alpha = .05$ ,  $1-\beta = 0.8$ ) with a large effect size  $f^2 = 0.38$  (Murphy, Myers, & Wolach, 2014), data from at least 21 animals (7 per a group) should be obtained.

## **Discussion**

The main objective of the present study was to investigate whether improved behavioural performance on a skilled reaching task observed following iTBS (Tang, Bennett, et al., 2015) shares a common neurophysiological mechanism with LTP changes induced by motor training alone. It was hypothesised that enhancement in performance following iTBS is facilitated by LTP-like mechanisms observed during use-dependant LTP. However, the data in the current thesis did not support this hypothesis.

The current project comprised of two aims. The first aim was to develop and optimise an immunofluorescent protocol for four biomarkers of neural plasticity: Synaptophysin, PSD-95, Parvalbumin and Calretinin. This aim was only partially achieved, as the anti-PSD-95 failed to bind its antigen target (see Appendix B). The immunofluorescent labelling for Synaptophysin produced an expected pattern of dense synaptic puncta marking active sites of excitatory presynaptic signalling. In contrast, the labelling of calcium-binding proteins Calretinin and Parvalbumin visualised the overall morphology of interneuron cells.

The second aim was to apply the optimised protocols to tissue samples obtained from animals in the Tang, Bennett, et al. (2015) study, quantify the abundance of immunofluorescently labelled proteins, and compare the amount of these proteins detected in relevant parts of the brain between the three groups. It was expected that any potential differences in the amount of the proteins of interest would be found in the areas of the brain that are involved in facilitating the performance on skilled motor reaching and/or were directly stimulated. Thus, the areas of interest were primary motor cortex and dorsal striatum of hemisphere contralateral to the dominant paw (Doyon et al., 2002; Whishaw & Pellis, 1990). No changes in abundance of proteins of interest were expected in the control area of olfactory processing (Watson et al., 2010) – piriform cortex in the hemisphere contralateral to the dominant paw.

It was predicted that the enhancement in performance on a skilled reaching task in animals that received iTBS, compared to the group with sham stimulation and handling group was facilitated by LTP-like neural mechanisms. Therefore, significant increase in Synaptophysin, and significant decrease in Calretinin and Parvalbumin was hypothesised in primary motor cortex and dorsal striatum of the animals that received iTBS, compared to the sham, and handling groups. There was no significant change in the expression of these markers expected in the piriform cortex.

Due to paraformaldehyde induced tissue degradation, data for Calretinin and Parvalbumin were not obtained. The results for Synaptophysin only partially supported the hypothesis. In line with the predicted outcomes, there was no significant difference found in the piriform area of the hemisphere contralateral to the dominant paw between the three groups (as indicated by inspection of 95%CI;



see Tables 8, 9, 10). However, contrary to predicted outcome, there was also no significant difference found in the primary motor cortex and dorsal striatum.

### **Synaptophysin in the Primary Motor Cortex**

The results indicate that in the primary motor cortex of the iTBS treated animals, there were fewer active synapses per  $0.01 \text{ um}^2$  of the neuropil, and these synapses were bigger, and occupied larger area of the neuropil, than active synapses in both sham and handling group. For all three experimental groups, the 95%CI around the mean were very wide with a substantial overlap, indicating no statistically significant difference between these scores (Cumming & Finch, 2005). Because our sample size was very small, and 95%CI are particularly sensitive to sample size (bigger sample, narrower interval), data were also inspected for overall trends. It was revealed that the differences in average scores between the three groups were very subtle for each measure (count, size, area coverage). For example, there was only 1% difference between the average percentages of the neuropil covered by puncta between the three groups (19% iTBS, 18% SHAM, 17% HAN; see Tables 4, 5, 6 and Figures 8, 9, 10 for more details). Therefore, it is possible to assume that even with an increased sample size, no meaningful differences would be found between the three experimental groups for this biomarker of synaptic activity.

### **Synaptophysin in the Dorsal Striatum**

Similar to the results from primary motor cortex, when compared to sham and handling groups, the dorsal striatum of iTBS treated animals had fewer active synapses per  $0.01 \text{ um}^2$ . However, in contrast to primary motor cortex, these synapses were also smaller, and occupied smaller area of the neuropil. These differences were again not assumed to be statistically significant, as suggested by the large overlap of 95%CIs (Cumming & Finch, 2005). Similar to the findings in motor cortex, the

overall differences in average scores between the tree groups was marginal on all three measures (see Table # and Figure #), therefore it is likely that the trend for smaller between the scores are not meaningfully different.

### **Synaptophysin in the Piriform Cortex**

Number of active synapses per  $0.01 \text{ um}^2$  was highest in the group with sham stimulation, followed by the iTBS group, and control group. The average size of the puncta was biggest in the control group, followed by control group and iTBS, and the average percentage of area covered by the puncta was highest in the sham group, followed by the control group and iTBS. Substantial overlap of 95%CI suggested that there was no statistically significant difference between the scores. Inspection of trends indicated only marginal differences in the average scores between the groups for all measures, suggesting there was also no meaningful difference between these scores. This finding that supports our hypothesis of no significant changes in the abundance of Synaptophysin in piriform area of hemisphere contralateral to the dominant paw.

### **Limitations**

Due to paraformaldehyde induced tissue degradation, four samples had to be excluded from the analysis, resulting in a restricted sample size of eight animals. Therefore, the generalisability of current findings is limited, and all results should be considered reflective of only the sample under the investigation. To avoid this methodological confound in the future, optimisation of fixation protocol is recommended. For example, Schneider Gasser et al. (2006) provide a detailed description of differential fixation protocols for detection of markers of synaptic plasticity, with suggested postfix in 2-4% PFA for 10-45min.

## **Theoretical Implications**

The findings of this study did not support the view that iTBS-induced enhancement of motor performance is facilitated by LTP-like mechanisms similar to that observed during use-dependant LTP on the presynaptic membrane. Our results also indicate that there were no remarkable differences in the abundance of Synaptophysin between all three groups, including the handling control group, and sham groups that performed skilled reaching training without stimulation. The most likely explanation for this finding relates to the process of homeostatic plasticity. Synaptic plasticity in form of LTP/LTD that was under investigation in this study can be conceptualised as a rapid and input specific form of plasticity, as it requires coordinated activity of pre- and postsynaptic cell (Caroni et al., 2012). In contrast, homeostatic plasticity refers to a slower compensatory process by which neurons regulate their excitability (Vitureira & Goda, 2013). As too much excitation can be detrimental, cells can regulate their overall excitatory input through a homeostatic process of synaptic scaling - increased excitation on a particular synapse, is accompanied by decrease of excitation on neighbouring synapses (Vitureira & Goda, 2013). Through this compensatory process the cell is able to provide efficient responses to incoming stimuli, while maintaining equilibrium of its overall excitatory input. Unlike synaptic LTP/LTD that occurs locally and rapidly (Xu et al., 2009), homeostatic neuroplasticity is slow acting process that produces lasting global changes (Vitureira & Goda, 2013).

Since the animals in the Tang, Bennett, et al. (2015) study were trained over 10 days period, it is feasible that a homeostatic process of synaptic scaling had taken place over the 10 days of testing (Figure 2). This is consistent with findings of Lee

et al. (2014), who reported significant up-regulation of Synaptophysin following single session of high-frequency stimulation (rapid LTP like response). However, following 10 sessions of the stimulation, there was no significant change between experimental and control group (possible homeostatic effects). In addition, similar to our findings, following 10 sessions of high frequency stimulation, the overall difference in average scores between the rTMS and control group were very subtle (Lee et al., 2014). This explanation would also be consistent with findings of Ma et al. (2013) who found no significant change in Synaptophysin following high frequency rTMS delivered over 20 days.

### **Future Directions**

Our findings are in agreement with those of Vlachos et al. (2012), who reported no significant change in presynaptic LTP marker Synaptophysin following high frequency rTMS. However, they also found an increase in postsynaptic markers of LTP, F-actin (microfilament protein involved in restructuring of postsynaptic spines) and GluA1 (subunit of AMPA receptors found on postsynaptic membrane of excitatory neurons). These findings suggest that rTMS affects pre- and postsynaptic sites of excitatory neurons differentially. Differential effects of high frequency rTMS was also reported on inhibitory interneurons (Benali et al., 2011). Excitatory rTMS was linked to increase in protein expression of Parvalbumin positive interneurons, but no change in expression of Calbindin positive interneurons (Benali et al., 2011). Differential effect of high frequency rTMS on diverse classes of neurons suggests that mechanisms of rTMS could be facilitated by the activity of local inhibitory circuits (Funke & Benali, 2011). Different subsets of interneurons preferentially connect onto specific sites of their output neurons (e.g. PV<sup>+</sup> interneurons preferentially synapse onto somatic and perisomatic sites of excitatory neurons),

creating a local inhibitory circuits (Chen et al., 2015). As demonstrated by Benali et al. (2011), not all subtypes of inhibitory cells react equally to rTMS, therefore it is possible that these differential effects on certain subtypes of interneurons would translate to differential effects of specific local inhibitory circuits along the excitatory cells. This theoretical reasoning would also be in line with recent findings of Chen et al. (2015), who demonstrated that differential effects of local inhibitory circuits on activity of excitatory neurons play a crucial role in motor-learning induced spine reorganisation.

Taken together, it would be of particular interest to further investigate effect of rTMS on the four markers proposed in this study (presynaptic Synaptophysin and postsynaptic PSD-95 of excitatory cells, and calcium-binding proteins of different subsets of inhibitory cells). Investigation of the effects of rTMS on particular subsets of excitatory and inhibitory cells could be of particular relevance in case of disorders related to the disturbance of excitatory-inhibitory balance in general activity of central nervous system . For example, deficits in neural plasticity are associated with neurodegenerative diseases such as Alzheimer's (Battaglia et al., 2007), or Parkinson's Disease (Suppa et al., 2011). Conversely, increased brain plasticity, is associated with unstable brain connectivity and maladaptive behaviours observed in conditions such as autism (Oberman et al., 2010) or schizophrenia (Hasan et al., 2011). In addition, a decrease in plasticity is also associated with maturational changes and cognitive decline due to aging (Sambataro et al., 2010). Therapeutic interventions using rTMS can be particularly effective and selective in its influence when combined with other appropriate therapies, practice currently applied to treatment of major depressive disorder (Cramer et al., 2011).

## **Conclusion and Contributions**

The contribution of the present study to research of synaptic plasticity following iTBS stimulation is twofold. Firstly, it was demonstrated that the detection of synaptic markers requires careful consideration of a paraformaldehyde fixation treatment, as this was found to interfere with the binding of the antibodies. In particular, the concentration of the PFA and duration of the PFA post-fixation were identified as most likely to influence the binding of the antibody to the antigen, as well as the level of autofluorescence within the tissue.

Secondly, the findings of the present study suggest that following 10 days of stimulation sessions and motor training, or motor training alone there was no significant difference in the number and size of the active excitatory presynaptic sites. This effect can be attributed to the homeostatic process of synaptic scaling. Although these findings are based on a small sample of animals, and have limited generalisability, it is suggested that any future investigation into the neurophysiological underpinnings of rTMS induced enhancement in motor performance, should consider tissue collection during the fast learning period, before the homeostatic processes take place.

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## **Appendix**

### **Phosphate Buffered Saline**

To make 1l of PBS dissolve in distilled water:

8.0 g NaCl

0.2g KCl

1.44g Na<sub>2</sub>PO<sub>2</sub>

0.24g KH<sub>2</sub>PO<sub>4</sub>

### **Cresyl Violet Staining**

#### **Reagents:**

Dissolve 2.5 g of powder in 10 ml Ethanol using mortar and pestle

Measure 500 ml dH<sub>2</sub>O with a cylinder

Pour the solution of CV in a 1000 ml beaker with the help of some of the dH<sub>2</sub>O previously prepared

Add the remaining water and boil under continuous stirring for few seconds

Let the solution cool down at RT

Adjust pH to 2.85 with ~ 10-15 ml of Acetic Acid (glacial)

Filter solution with paper filter

#### **Procedure:**

Deep sections on slide in distilled water: 2x2 min

Cover with cresyl violet for 20-30 min

Dehydrate with alcohol 70 % (2x5 min), 90-95 % (2x5 min), 100 % (1x5 min)

Pass through Xylene (2x5 min)

Mount with Depex

### **Auto-fluorescence quenching**

Incubate 0.25% potassium permanganate ( $\text{KMnO}_4$ ) in PBS at RT for 20mins)

2 x 2min PBS wash

Sections will have a brown colour

1% potassium metabisulfite ( $\text{K}_2\text{S}_2\text{O}_5$ ) in 1% oxalic acid in PBS for approximately

2mins (time depends on when brown colour has cleared)

3 x 10mins PBS wash at RT on orbital shaker

### **Antigen Retrieval**

#### **Reagents:**

Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)

Tri-sodium citrate 2.94g

Distilled water 1000ml

Mix to dissolve, adjust pH to 6.0

#### **Procedure**

Preheat water bath containing heat proof dish with Sodium Citrate Buffer to 95 °C

Immerse brain sections into the dish for 20mins

Remove from bath and allow to cool down to room temperature